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Examination of the Taxonomy and Diversification of *Leontopithecus* using the Mitochondrial Control Region

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Abstract *Leontopithecus* comprises 4 taxa: black lion tamarins *(L. chrysopygus)*, golden lion tamarins (*L. rosalia*), black-faced lion tamarins (*L. caissara*), and golden-headed lion tamarins (*L. chrysomelas*). Endemic to the Atlantic Forest of Brazil, they are endangered (Appendix I, CITES; IUCN Critically Endangered: *Leontopithecus chrysopygus, L. caissara*; IUCN Endangered: *L. rosalia, L. chrysomelas*). The 4 taxa are differentiated morphologically and geographically and occupy different habitat types. However, it is not clear if all of them are separate species, particularly *Leontopithecus caissara*, or how they are related to one another evolutionarily. Therefore, we investigated lion tamarin differentiation and radiation. We sequenced the mtDNA control region and performed phylogenetic analyses, population aggregation analyses, and Mantel tests for geographic/genetic correlation. Mitochondrial genetic data suggest 3 distinct lion tamarin clades *(Leontopithecus chrysomelas; L. caissara;* and *L. chrysopygus/L. rosalia*). Phylogenetic analysis also supports: 1) the basal lion tamarin is *Leontopithecus chrysomelas*, and not *L.*

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chrysopygus, 2) *L. caissara* is not subspecific to *L. chrysopygus*, and 3) Quaternary forest refugia may have shaped lion tamarin diversification via a pattern that does not follow the theory of metachromism. Even though mitochondrial genetic analyses do not unequivocally support the 4 lion tamarins as separate species, one should consider the 4 lion tamarins, with equal conservation priority based on the combination of morphological, genetic, and habitat differentiation. Each of them is an extremely valuable flagship species that focuses attention on the diminishing, highly endemic Atlantic Forest of Brazil.

Keywords *Leontopithecus* · mitochondria · phylogenetic · refugia · taxonomy

Introduction

Leontopithecus comprises 4 taxa, which are easily distinguished using chromatic patterns: black lion tamarins (*Leontopithecus chrysopygus*), golden lion tamarins (*L. rosalia*), golden-headed lion tamarins *(L. chyrsomelas*), and black-faced lion tamarins (*L. caissara*). They are endangered and allopatrically distributed across remnant fragments of the Atlantic Forest of Brazil (Fig. 1), and each of them occupies a unique habitat with respect to altitude, vegetation, and climate (Coimbra-Filho 1976; Coimbra-Filho and Mittermeier 1973; Hershkovitz 1977; Joly *et al.* 1991; Lima *et al.* 2003; Keirullf *et al.* 2002; de Pinto and Rylands 1997; Rizzini *et al*. 1988; Rohe *et al.* 2003; Valladares-Padua 1987; Veloso 1966). Their allopatric distributions may be historical, not anthropogenic, though their pre-Columbian distribution is not certain (Kinzey 1982).

The taxonomic status and evolutionary history of lion tamarins remain unclear. We helped clarify the taxonomic and evolutionary uncertainty surrounding the lion tamarins by phylogenetically analyzing their mtDNA control region (D-loop) sequences.

Taxonomic Status

Currently, 4 lion tamarins are recognized as separate species (Seuánez *et al*. 2002). Researchers assigned specific status to 3 of them —*Leontopithecus chrysomelas, L. chysopygus,* and *L. rosalia*— based on morphometric cranial, mandibular, and dental characters (Burity *et al.* 1999; Della Serra 1951; Natori 1989; Natori and Hanihara 1989; Rosenberger and Coimbra-Filho 1984). Forman *et al.* (1986) questioned the species status of these 3 more common species because of low levels of allozyme differentiation. However, the 3 species formed reciprocally monophyletic groups in a phylogenetic analysis of the interphotoreceptor retinol binding protein (IRBP) intron (Mundy and Kelly 2001).

The scarcity of morphological studies and lack of published genetic studies leave the taxonomic rank of *Leontopithecus caissara* very uncertain. Coimbra-Filho (1990) ranked it as a subspecies based on the discovery of individuals intermediate in coat color between *Leontopithecus caissara* and *L. chrysopygus* (Rylands *et al.* 1993). Burity *et al.* (1999) thought cranial and mandibular measurements suggest specific status for *Leontopithecus caissara*, but they admitted that the results are provisional owing to small sample sizes.

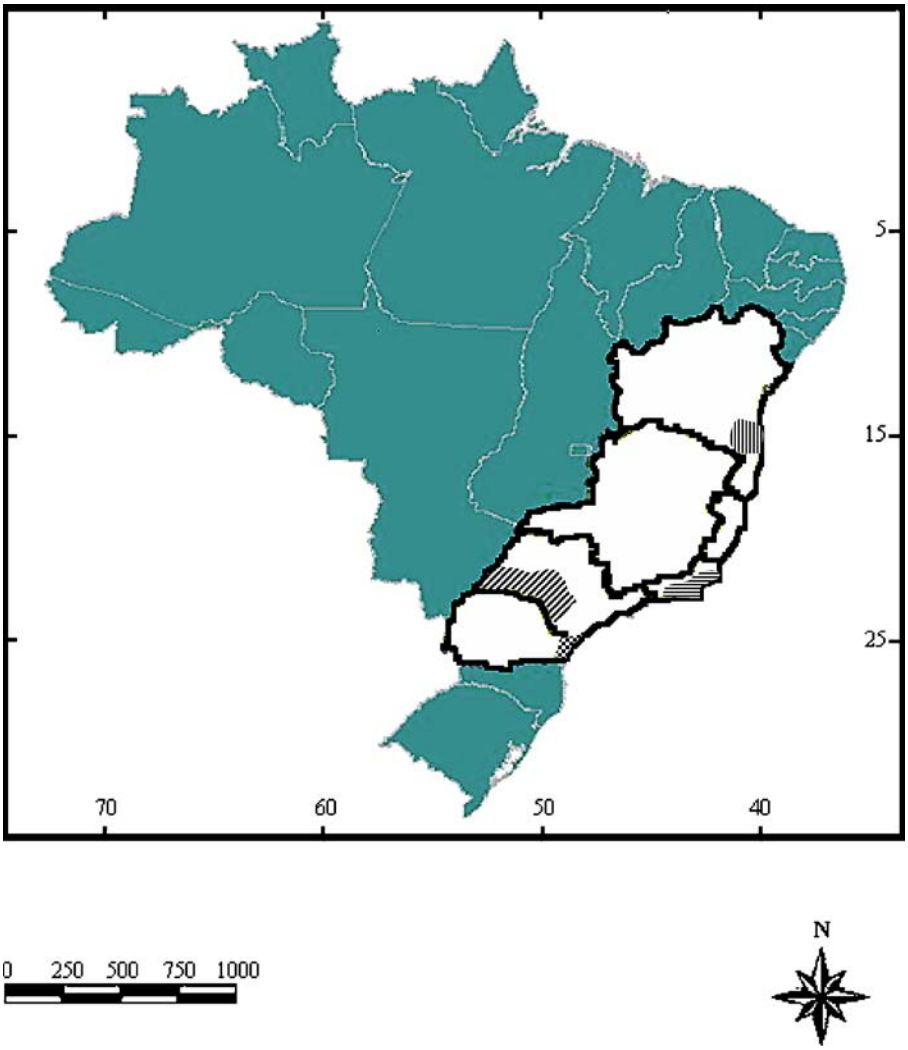

Fig. 1 Map of the distribution of *Leontopithecus* (modified from Rylands *et al*. 1996). Vertical lines, *Leontopithecus chrysomelas*; horizontal lines, *L. rosalia*; diagonal lines, *L. chrysopygus;* hatched, *L. caissara*. The white area highlights the states of Brazil containing the Atlantic Forest.

Evolutionary History

Because no fossil record exists for *Leontopithecus*, the determination of evolutionary relationships relies solely on extant samples. Some hypotheses relate *Leontopithecus chrysopygus* to *L. rosalia*, while others relate *L. chrysopygus* to *L. chrysomelas*. Natori (1989) grouped *Leontopithecus chrysopygus* with *L. rosalia* and apart from *L. chrysomelas* (Fig. 2a) based on 1 synapomorphy, among several dental and cranial characters he analyzed, which coincides with findings of Snowdon *et al.* (1986) based on phonetic analysis of vocalizations and Mundy and Kelly (2001) based on

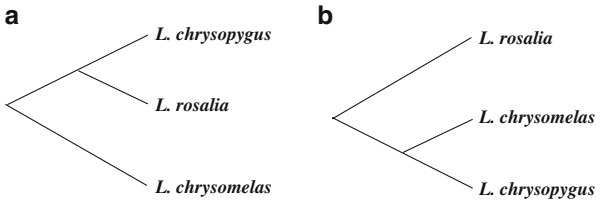

Fig. 2 Relationships hypothesized for 3 of the 4 species. (**a**) *Leontopithecus chrysopygus and L. rosalia* as sister taxa (Mundy and Kelly 2001; Natori 1989; Snowdon *et al*. 1986,). (**b**) *Leontopithecus chrysomelas* and *L. chrysopygus* as sister taxa (Burity *et al*. 1999; Rosenberger and Coimbra-Filho 1984).

phylogenetic analysis of the *IRBP* gene. In contrast, researchers grouped *Leontopithecus chrysopygus* and *L. chrysomelas* together apart from *L. rosalia* (Fig. 2b) in some studies based on shared morphological characteristics such as skull dimensions, neurocranial and facial components, and the size of the ascending mandibular ramus (Burity *et al.* 1999; Rosenberger and Coimbra-Filho 1984).

Hershkovitz (1977) proposed that *Leontopithecus chrysopygus* is most like the ancestral lion tamarin based on his theory of metachromism (1968) that states pelage pattern evolves in a linear, orthogenetic manner from dark (saturated) to light (bleached) forms. Rosenberger and Coimbra-Filho (1984) suggested the same evolutionary position of *Leontopithecus chrysopgygus* based on morphological characters though they note that their analysis was preliminary. Conversely, other morphological and IRBP genetic data support *Leontopithecus chrysomelas* as most like the ancestral lion tamarin (Mundy and Kelly 2001; Natori 1989).

Hershkovitz (1977) also suggested that metachromism was the primary mechanism influencing lion tamarin diversification, while recognizing that forest refugia also may have played a role in their differentiation. Other researchers suggest that the genus differentiated primarily as a consequence of allopatric isolation among populations due to Quaternary shifts in climate and forest distribution (Kinzey 1982; Rylands *et al.* 1996). An alternative hypothesis to allopatric speciation is differentiation through isolation by distance of a continuously distributed group of organisms, resulting in a cline of variation.

We incorporated genetic and nongenetic criteria to help delineate lion tamarin taxonomy and evolutionary history. In the first part of the article, we use population aggregation (Davis and Nixon 1992) and phylogenetic analyses of the mitochondrial D-loop to diagnose lion tamarin taxa using criteria from the Phylogenetic Species Concept (Cracraft 1983) and Evolutionary Significant Units (Cracraft 1983; Moritz 1994). We then interpret results of genetic analyses with respect to known ecological and morphological data in accordance with both the cohesion species concept, which includes nonheritable data to identify unique taxa (Templeton 1989, 2001) and the character concordance approach, which requires congruence of several independent characters to identify taxonomic uniqueness (Avise and Ball 1990; Wilson and Brown 1953). In the second part of the article, we present lion tamarin evolutionary relationships and assess the fit of the data to the theories of diversification, including metachromism and isolation by distance.

Materials and Methods

Samples

We collected samples of wild *Leontopithecus chrysopygus* in the forests of the state of São Paulo via the methods of Valladares-Padua (1987). We collected blood, hair, and fecal samples from 6 of the 8 forest fragments that harbor *Leontopithecus chrysopygus* in São Paulo. We stored blood and fecal samples in 1:2 and 1:9 sample–to-buffer (100 mM EDTA, 100 mM Tris-base, pH 8.0, 1% sodium dodecyl sulfate [SDS]) ratios, respectively. We stored hair in desiccator beads (silica). We used samples that others provided from wild *Leontopithecus caissara* captive and wild *Leontopithecus rosalia* and from captive *L. chrysomelas,* as well as captive *Callithrix* and *Saguinus* (Table I). In the case of *Leontopithecus caissara*, the samples came from dried feces from unfavorable (humid) storage conditions.

Laboratory Methods

We extracted DNA from tissue and fecal samples via the Qiagen Tissue Kit procedure (Qiagen, Valencia, CA, no. 69506). Unlysed fecal sediment settled to the bottom before we transferred the solution to the Qiagen filter apparatus. We subjected the samples twice to the Qiagen process, with omission of the initial lysis steps in the second Qiagen round, and eluted DNA in water or 1:10 TE (100 μM EDTA, 1 mM Tris-HCL, pH 8.0).

We initially used primers 283 (5′-TACACTGGTCTTGTAAACC-3′) and 282 (5′-AAGGCTAGGACCAAACCT-3′) from Jacobs *et al.* (1995) to amplify the D-loop and to obtain sequences for *Leontopithecus* and the outgroups. We then used primers designed specifically for our study (Table II), to amplify the entire D-loop in most samples and only hypervariable region I in some fecal samples of *Leontopithecus caissara* that were highly degraded. We amplified all samples, except the degraded

Table I Sample list, provider, and number of samples used in study

Species	Code	Provider(s)	Sample Number
Leontopithecus chrysopygus	Lchy	1. Dr. Vern McGrann: Ft. Worth Zoo. 2. Collected by Perez-Sweeney 3. Dr. Lisa Forman: Cell Diagnostics	11 (from 6 forest fragments)
Leontopithecus caissara	Lca	Fabiana Prado, IPÊ (Institute for Ecological Research, Brazil)	4
Leontopithecus rosalia	Lro	Dr. Lisa Forman: Cell Diagnostics	7
Leontopithecus chrysomelas	Lche	1. Dr. Lisa Forman: Cell Diagnostics 2. Genbank: Lch108, accession no. U89011 (Tagliaro *et al.* 1997)	8
Callithrix pygmea	Cpy	Dr. Amanda Goudy: Wisconsin Regional Primate Center	1
Callithrix jacchus	Cja	1. Dr. Lisa Forman 2. Genbank: Cja33 U86526 OR Cja43 U88840 (Tagliaro *et al.* 1997)	2
Saguinus oedipus	Soe	Elizabeth Curran: New England Regional Primate Research Center	1

Table II Designed primers

PCR primers	
LEONDLF1	AAC TAA TTC TAC CAT CAA CAC CCA AAG CT
LEONDLR1	GTT TCA GTA TAA CCA AGC CCT GTC TAT ATG
LEONDLR2	TCA TGT CCT GTA ACC ATT AAC TTG ATA TCC ACT
LEONDLR3	GTC TTG CTT TAA ACT TAA TCT ACA TTA ACT
Internal sequence primers	
ILEONDLF1 (for *Leontopithecus* individuals only)	AAC ATG CTC AAC CCT AGG A
ILEONDLR1NW (for outgroup individuals and *Leontopithecus chrysomelas* only)	GGC GCG ATG ATA GCA TAA AGT C
ILEONDLF1DNW (for outgroup individuals only)	GGT CTC TTA ATC TAC CAA CCT ACG
ILEONDLF2 (targets R domain minus poly A,T rich region)	AAT GTA CTC ATC AGC ATC G
ILEONDLF3t4 (for cloned sample only to target poly A,T rich region and rest of R domain)	GGT AAA AAC ACC TTT TT

We used 2 (LEONDLR1, LEONDLR2) of the 3 reverse PCR primers to amplify shorter regions. We also used the reverse primers as internal sequence primers for *Leontopithecus rosalia*, *L. chrysopygus*, and *L. caissara*. All primers are in the 5′ to 3′ direction.

DNA samples from dry feces of *Leontopithecus caissara*, in 2.5 mM Mg^2, 10 mM Tris-HCl, 50 mM KCl, 0.2 mM dNTPs, and 1.0 U of *Taq* in a 50-μl reaction mixture. We amplified the DNA from dry fecal samples in 4 mg/ml of bovine serum albumin (BSA), 1.4 mM Mg^2, 9.4 mM Tris-HCl, 46.8 mM KCl, 0.1 mM dNTPs, and 2.0 U of *Taq* in a 25-μl reaction (Prithiviraj Fernando, Columbia University, *pers. com.*). We initiated the polymerase chain reaction (PCR) process with a hot start at 95°C for 6 min (by either withholding *Taq* polymerase during the 6-min incubation or by using Taq Gold [PerkinElmer, Waltham, MA, no. N808–0240]). Then for all samples except degraded DNA, we ran the PCR for 35 cycles of 94°C at 1 min, 60°C at 45 s, and 72°C at 45 s per cycle. We subjected degraded DNA from dry fecal samples to the same temperature cycles, but for 45 cycles, subsequently run on an agarose gel, punched from the gel, and then reamplified under the same initial conditions for 35 cycles.

We cleaned amplified products with a Qiagen PCR purification kit (Qiagen no. 28106). We conducted cycle sequencing reactions on a PerkinElmer thermal cycler with the FS-DNA sequencing kit (PerkinElmer no. 402079) and rid the sequencing products of excess dyes with CentriSep Spin Columns (Princeton Separations, No. CS-901). We used an ABI 377 PRISM automated sequencer (PerkinElmer) to generate sequence data from the reaction products.

Because Moreira and Seuánez (1999) and Mundy *et al.* (2000) found nuclear inserts of mtDNA sequences (numts) in New World monkeys, we took measures to ensure that our amplifications did not include nuclear inserts. First, we designed primers specifically for our study. Second, we produced sequences from total DNA amplifications via the designed primers and checked them for double peaks. Even though clean sequences were produced from total DNA amplifications, a more

definitive method was needed to ensure that the designed primers targeted the mtDNA D-loop and not a nuclear insert (Collura and Stewart 1995; Mundy *et al.* 2000).

Therefore, we isolated mitochondria from tissue of *Leontopithecus chrysomelas*, *Callithrix jacchus,* and *Saguinus oedipus*, and subjected the organelle isolates to DNase to eliminate extraneous nuclear DNA from each sample (Mecocci *et al.* 1993). We tested the samples for lack of visible nuclear DNA amplification by subjecting the samples to PCR reactions with nuclear primers and testing for detection on an agarose gel. From samples showing a lack of nuclear amplification, we designed primers to amplify the desired mtDNA locus (Table II). We compared the sequence from organelle isolates to D-loop sequences from total DNA preparations of the same individuals with the same primers. Because sequences from total DNA were the same as those for organelle DNA, we concluded that the primers were likely selectively targeting mtDNA in the total DNA extract.

We designed internal sequencing primers for *Leontopithecus* and the outgroups (Table II) and sequenced the entire D-loop for 1 black lion tamarin individual (Fig. 3). To obtain a readable sequence of the poly(A,T)-rich region, we cloned the D-loop PCR product into a TA cloning vector (Invitrogen) before sequencing that region.

Phylogenetic Analysis

We focused on the left domain (hypervariable region I) and part of the central domain of the D-loop for all samples because the left domain contains the most informative characters in the taxa examined, while the right domain sequences from the 3′ end of the D-loop showed variation mostly in the repeat regions and did not align at all to our outgroups (data not shown).

We aligned proofread sequences via ClustalW first, then by eye (Fig. 4). Some alignment ambiguous areas between the outgroup and the ingroup are evident owing to the high level of divergence between the outgroups (*Saguinus* and *Callithrix*) and *Leontopithecus*.

We performed all phylogenetic analyses using maximum parsimony, maximum likelihood, and distance methods in PAUP (version 4.b7; Swofford 1991). We executed phylogenetic analysis with alignment ambiguous sites included and excluded to determine their effect on parsimony, maximum likelihood and distance analyses (*cf.* Gatesy *et al.* 1993). In addition, once we identified a basal lion tamarin sequence unequivocally, we performed phylogenetic analysis using the basal tamarin as the outgroup for the rest of the lion tamarins.

We performed maximum parsimony analysis with and without weighting schemes. Because the D-loop experiences a significant amount of rate heterogeneity as evidenced by different substitution rates for different sites (Excoffier and Yang 1999; Meyer *et al.* 1999), we assigned larger weights to the more slowly evolving sites. We did this by first estimating the rate of substitution per site in MacClade (Maddison and Maddison 1992), using a parsimonious consensus tree based on an unweighted, unaltered data set (Fig. 5). Then, in a successive weighting scheme, we took the inverse of the rates, assigned them as weights, and performed a subsequent parsimony analysis. In addition, we down-weighted the signal from highly mutable

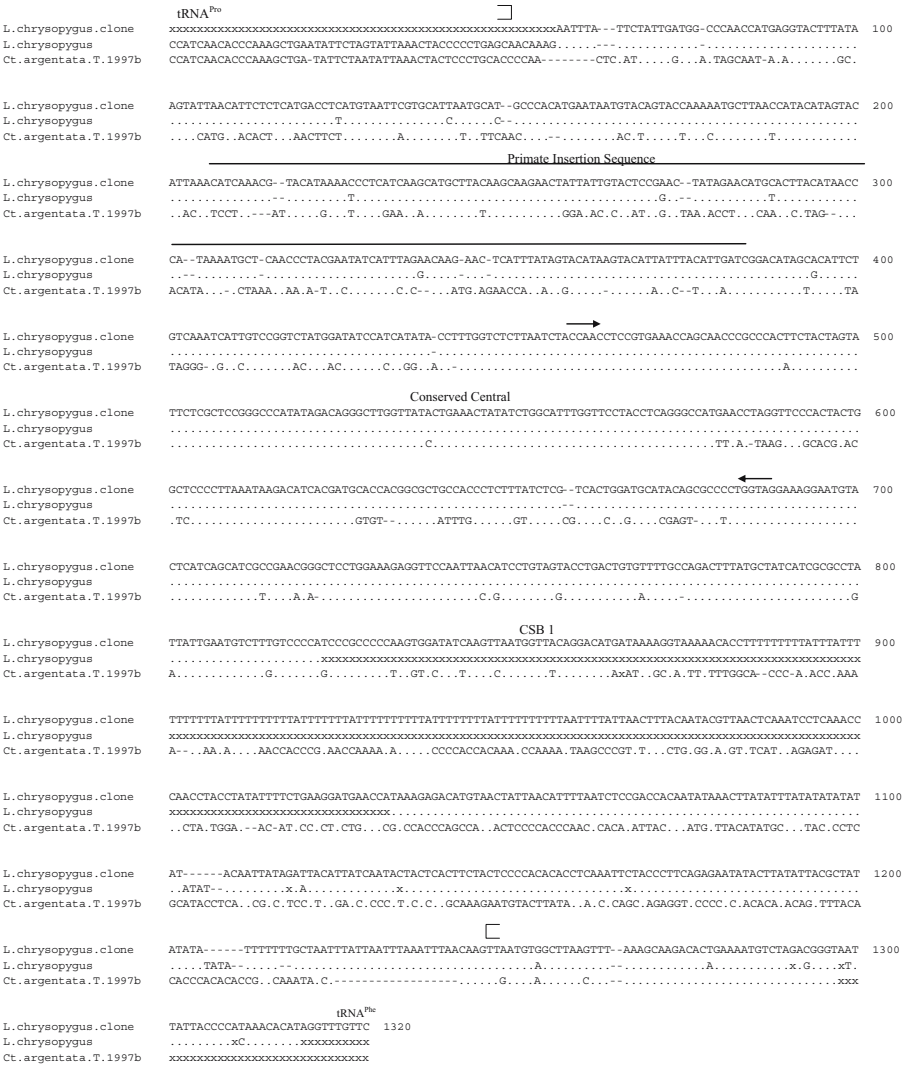

Fig. 3 Sequence of the entire control region of a *Leontopithecus chrysopygus* individual [the PCR product had to be cloned to obtain poly(A, T)-rich region], and a partial sequence of another *L. chrysopygus* excluding the poly(A, T) region. Straight line, primate insertion sequence; right bracket, end of the tRNA proline flanking the 5′ part of the D-loop; left bracket, beginning of the tRNA phenylaline; arrows, conserved central domain; dashed line, conserved sequence block 1 (CSB 1) region.

sites by reweighting the characters with respect to the retention index, a measure of homoplasy (*cf.* Archie 1996) in parsimony analysis.

We performed maximum likelihood analyses under the K81uf model with a γ estimation for rate heterogeneity as chosen by Modeltest (Posada and Crandall 1998). We eliminated *Saguinus oedipus* from the maximum likelihood analysis because its sequence contains large gaps in alignment, and thus would have forced

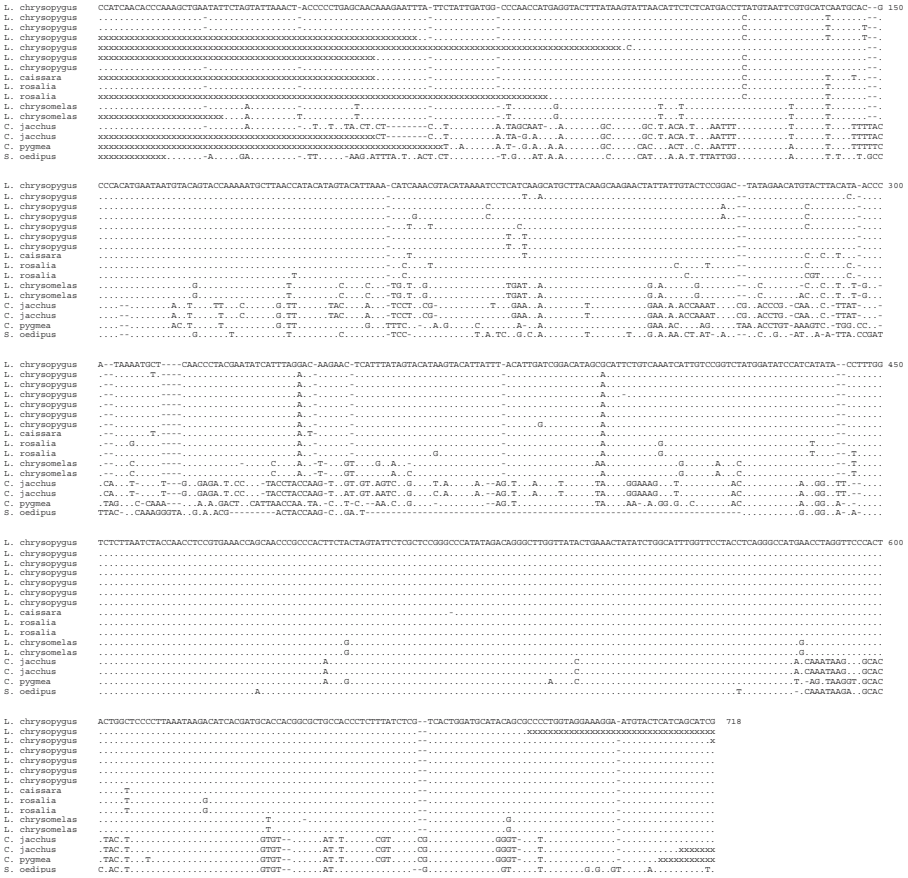

Fig. 4 Aligned sequence data set used in our study. Only the variable individuals are shown; i.e., each individual represents a single haplotype.

the exclusion of some characters from the analysis. We executed maximum likelihood analysis via subtree pruning and heuristic sampling.

We performed neighbor-joining analysis (Saitou and Nei 1987) via the Tamura and Nei (1993) distance that takes into account unequal base substitution rates and using the raw distances (the distance calculated from the data set) for comparative purposes.

Taxonomic designation and diversification

To address taxonomic designations, we analyzed the phylogenetic tree for monophyly and reciprocal monophyly. In addition, we performed population aggregation analysis manually (Davis and Nixon 1992). Population aggregation analysis groups individuals according to shared characters that are constant

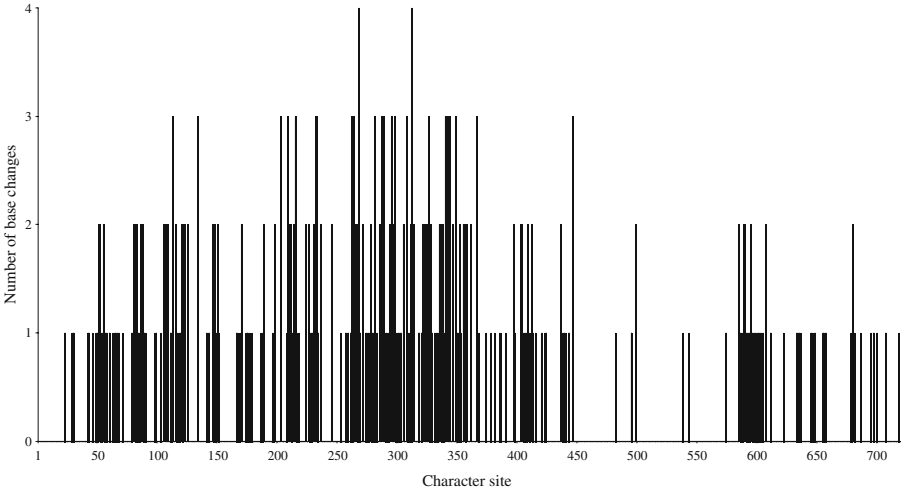

Fig. 5 Nucleotide substitution rates at each site calculated in MacClade and based on a consensus maximum parsimony tree constructed from unweighted sequence data. The *y*- axis represents the number of mutations for each site. The *x*-axis represents each site.

(invariable) in a putative taxon and unique to that taxon versus other congeneric taxa. We then interpreted our data in light of morphological and habitat differentiation to identify taxonomic status.

To investigate diversification of lion tamarins, we examined the phylogenetic relationships we identified in our study in light of Pleistocene climatic changes, hypothesized forest refugia, and current and past geographic distributions. We performed a Mantel test between genetic distance and geographic distance to test for evidence of clinal variation and isolation by distance.

Results

Mitochondrial D-loop sequence

While 718 bp are in Fig. 4, exclusion of missing data left 582 bp. We found 16 haplotypes from the 34 individuals in Table I.

The transition-transversion ratio from the raw D-loop data is *ca*. 1:1 including the outgroups and 5:1 excluding the outgroups. Estimates from maximum likelihood were on the order of 2.5:1 with the outgroups and 7:1 without the outgroups.

Taxonomy and evolutionary relationships

All trees show monophyly for *Leontopithecus caissara* and *L. chrysomelas*. Monophyly for *Leontopithecus caissara* may be constrained by sample size because we found only 1 haplotype. *Leontopithecus chrysopygus* was either paraphyletic with respect to *L. rosalia* (Fig. 6c), or formed a polytomy with *L. rosalia* (Fig. 6a) in

nonweighted parsimony and in likelihood analyses. The analyses that resulted in reciprocal monophyly for *Leontopithecus chrysopygus* and *L. rosalia* were maximum parsimony analyses via 2 weighting schemes (successive weighting with retention index and weighting sites for rate heterogeneity) and neighbor-joining analyses via raw and Tamura-Nei distances (Fig. 6b). Only 1 site differentiated

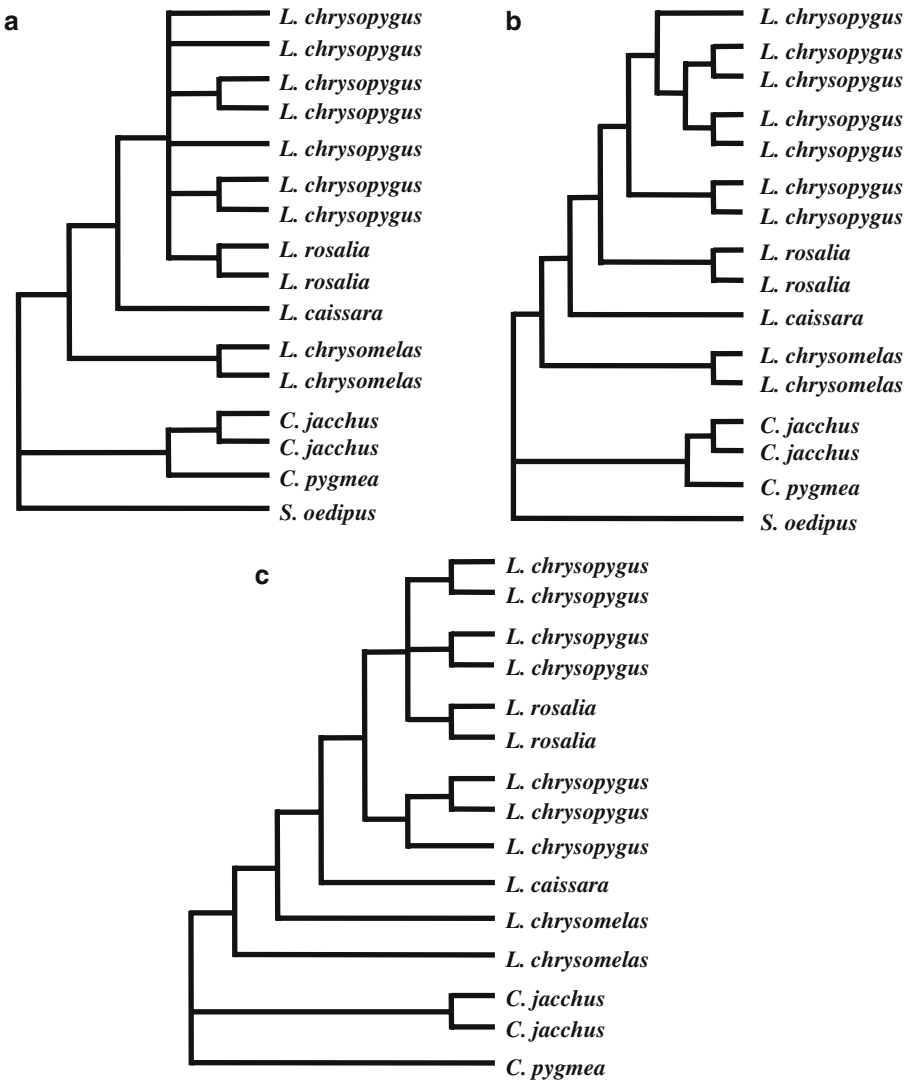

Fig. 6 Phylogenetic trees of *Leontopithecus*. Each tree is supported by >1 analysis. (**a**) We constructed the tree under maximum parsimony via 2 schemes: no weight, alignment ambiguous site included; and no weight, but alignment ambiguous sites excluded. (**b**) We constructed the tree under maximum parsimony via 2 schemes: retention index and rate heterogeneity weights; and neighbor-joining via 2 schemes: raw distance; and Tamura and Nei distance. (**c**) We constructed the tree under maximum likelihood under 2 schemes: alignment ambiguous excluded; and no exclusion.

Leontopithecus chrysopygus from *L. rosalia* (a transition from C to T) in the weighted parsimony analyses. Trees that we constructed with *Leontopithecus chrysomelas* as the outgroup and subjected to the aforementioned analyses provided no additional phylogenetic information (data not shown). From the mitochondrial phylogenetic trees, 3 distinct clades are evident: *Leontopithecus chrysomelas*, *L. caissara*, and *L. rosalia*/*L. chrysopygus*.

Under a population aggregation analysis, only *Leontopithecus caissara*, *L. rosalia,* and *L. chrysomelas* show diagnosable characters. *Leontopithecus chrysopygus* shows diagnosable characters if *Leontopithecus chrysomelas* is excluded.

Regardless of analytical methods, all trees showed *Leontopithecus chrysomelas* basal to the other *Leontopithecus* taxa. No tree showed *Leontopithecus caissara* sister to *L. chrysopygus,* but instead showed a close relationship between *L. chrysopygus* and *L. rosalia*.

Diversification and clinal association

We compared the intertaxonomic mean genetic distances to the shortest straight-line geographic intertaxonal distances to test for diversification via isolation by distance. The mean genetic distance and geographic distance provided some level of correlation (Mantel test, $r=0.73$), but not significantly so ($p=0.26$, in a randomization 1-tailed test for significance for 1000 permutations). Though not statistically significant, 53% of the variance (r^2) in genetic distance is explained by geographic distance, in part owing to *Leontopithecus chrysomelas* being the most geographically distant and genetically divergent taxon from the other 3 taxa. However, *Leontopithecus chrysomelas* is geographically closer to *L. rosalia* and farthest from *L. caissara*, yet its genetic distance to *L. caissara* is smaller than the comparable genetic distance to *L. rosalia*.

The phylogenetic trees also do not map well onto the geographic distance data. Again, the most closely related taxon to *Leontopithecus chrysomelas* is *L. caissara*, even though *L. caissara* is the most geographically distant to *L. chrysomelas*. In addition, even though genetic distances are smallest between *Leontopithecus caissara* and *L. chrysopygus*, they seem not to be the most closely related (Fig. 6). In fact, *Leontopithecus chrysopygus* is more closely related to the geographically more distant *L. rosalia* than it is to *L. caissara*.

Discussion

We present 3 unambiguous clades revealed by phylogenetic analysis of mtDNA control region sequences: *Leontopithecus chrysomelas, L. caissara,* and *L. chryspopygus/L. rosalia*. *Leontopithecus chrysomelas* occupies a basal phylogenetic position and is the most divergent of the lion tamarins.

Our mitochondrial data do not support the hypothesis of lion tamarin differentiation as a product of isolation-by-distance, nor do they support Hershkovitz's hypothesis (based on his theory of metachromism) that the “ancestral lion tamarin may have been nearly or entirely black,” making *Leontopithecus chrysopygus* “nearest the ancestral form” (1977, p. 825). Lion tamarins may have

diversified allopatrically as a product of Quaternary climatic fluxes without constraints from Hershkovitz's principles of metachromism. The phylogeny of lion tamarins suggests that the first lion tamarin taxon isolated was in Kinzey's (1982) Bahia refuge, where *Leontopithecus chrysomelas* lives today. The other lion tamarins may have lived in Muller's (1973) Paulista subcenter, which then fragmented into 1 refuge with *Leontopithecus caissara* and another with *L. chrysopygus* and *L. rosalia* and that refuge subsequently divided further to form the Orgãos refuge containing *L. rosalia* only.

Taxonomic Status

Our mtDNA phylogenetic data support specific status for *Leontopithecus chrysomelas*, in agreement with the nuclear genetic (Mundy and Kelly 2001) and morphological data (Burity *et al.* 1999; Della Serra 1951; Natori 1989; Natori and Hanihara 1989; Rosenberger and Coimbra-Filho 1984). Our phylogenetic data also support specific status for *Leontopithecus caissara*, in agreement with the preliminary analysis of Burity *et al.* (1999) and not subspecific status to *L. chrysopygus* as proposed by Coimbra-Filho (1990).

Under PAA, *Leontopithecus chrysopygus* is the only species that does not have unique characters that set it apart from the other lion tamarins. We sampled *Leontopithecus chrysopygus* the most and it exhibited a large number of haplotypes as compared to the other taxa. Therefore, it is reasonable to question whether the characters found for *Leontopithecus caissara* and *L. rosalia* under PAA would remain uniquely associated with them if more individuals were haplotyped. Conversely, it is expected that many of the defining characters of *Leontopithecus chrysomelas* would remain unique even in the face of more haplotypes given how divergent it is from the other lion tamarins.

Leontopithecus chrysopygus is para- or polyphyletic to *L. rosalia* without diagnosable characters in most analyses, and when it is monophyletic, it is distinguished from *L. rosalia* by just one nucleotide base (Fig. 6). One may argue that the lack of character concordance for mtDNA trees means a lack of specific status support for these two lion tamarin taxa, especially because there is a lack of distinction for *Leontopithecus chrysopygus* under PAA. However, the IRBP gene and morphological data support separate specific status for *Leontopithecus chrysopygus* and *L. rosalia*. In addition, the taxa occupy distinct habitats, which adds further support for taxonomic uniqueness under the Cohesion Species Concept (Templeton 1989, 2001). Thus, taking a character concordance approach across a wide set of data (Avise and Ball 1990; Wilson and Brown 1953), the combination of genetic, habitat, and morphological distinctions suggests that both taxa may continue to be seen as separate species and conservation units with equal status to the other lion tamarins.

Evolutionary History

Our data support the same basal status of *Leontopithecus chrysomelas* present in a nuclear genetic (Mundy and Kelly 2001) and a morphological study (Natori 1989). Our data do not support the hypothesis of *Leontopithecus chrysopygus* being closest

to the ancestral form, which was previously based on the presence of "primitive" features like a "relatively large and unadorned $I^{1,2}$", a "relatively tall $I_{1,2}$", and a "relatively massive premaxilla" (Rosenberger and Coimbra-Filho 1984, p. 167), and on the theory of metachromism (Hershkovitz 1977).

Our results show *Leontopithecus caissara* as distinct, but sister to the *L. rosalia/L. chrysopygus* clade, and in trees showing distinction between *L. chrysopygus* and *L. rosalia*, *L. caissara* still does not group with *L. chrysopygus*, even though Coimbra-Filho (1990) found individuals intermediate in dorsal coat color to *L. chrysopygus* and *L. caissara* (Rylands *et al.* 1993). Interestingly, *Leontopithecus rosalia* seems to have taken the expected position of *L. caissara* in that it is virtually indistinguishable from *L. chrysopygus* in our phylogenetic trees. Natori (1989) had similar results, in which *Leontopithecus chrysopygus* was united to *L. rosalia* by 1 synapomorphic character (a round P^2), and in vocalizations, Snowdon *et al.* (1986) showed that the 2 taxa were most similar to each other. The lack of a sister relationship between *Leontopithecus caissara* and *L. chrysopygus* also corresponds to cranial and mandibular data (Burity *et al*. 1999).

Metachromism

Hershkovitz (1977) proposed that the primary evolutionary path exhibited by each of the 3 isolated lion tamarin groups (excluding *Leontopithecus caissara*) "appears to have been a switch from eumelanin to pheomelanin production" (1977, p. 825), though he recognized that switching could occur in either direction between the 2 pathways (1968). Specifically, Hershkovitz (1977) provided the following course of lion tamarin diversification: lion tamarin coat color was derived from an agouti pattern, then moved to eumelanin and subsequently moved to a "progressive switching from eumalanin to pheomelanin," creating a process which "begins with the expression of reddish orange on the rump and thighs of the dominantly black *Leontopithecus rosalia chrysopygus*,…[and that expression of reddish/orange and golden] advances to the forequarters and parts of the tail of *Leontopithecus rosalia chrysomelas*, and [then] becomes generalized in the dominantly golden *Leontopithecus rosalia rosalia*." (p. 827). We showed 1 example of a orthogenetic switch from black (eumelanin) to orange (pheomelanin) in the dorsal region of a black lion tamarin that follows the scenario of color change at the population level (Fig. 7). However, our phylogenetic study, in accordance with Mundy *et al.* (2001), shows that *Leontopithecus chrysomelas*, with golden/orange limbs and head, and a black dorsum, is basal to the other lion tamarins as opposed to the mostly black *L. chrysopygus*. Thus, the black in *Leontopithecus chrysopygus* represents a reversal from the gold to black in certain chromogenetic fields, contrary to Hershovitz's proposal for lion tamarin diversification. It is not surprising that the principle of metachromism does not apply in the orthogenetic way envisioned by Hershkovitz (1977) because several mechanisms exist for a switching between the eumelanin and pheomelanin pathways (Jackson 1997; Newton *et al.* 2000; Ito and Kazumasa 2003; Hirobe *et al*. 2006). The biochemical pathways of eumelanin and pheomelanin are more complicated than Hershkovitz (1968) presented (Bradbury and Fabricant 1988).

Several researchers cautioned against using the principle of metachromism interspecifically to discern phylogenetic diversification (Jacobs *et al.* 1995; Shedd

Fig. 7 *Leontopithecus chrysopygus* individual from a forest fragment with a patched gold and black dorsal color pattern in contrast to the black body with orange/gold rump characteristic of *Leontopithecus chryspygus*.

and Macedonia 1991) because it enlists an orthogenetic mode of evolution that is more likely to lead to parallelisms among taxa than to divergence. A more likely explanation to the diversification of lion tamarins would be that the ancestral population displayed variation in coat color, and that certain patterns became fixed by chance in isolated populations during the Quaternary period.

Allopatric diversification and forest refugia

Historical data suggest that the allopatric distribution of lion tamarins before forest destruction was not that different from today, except that habitats were less fragmented and the distribution of *Leontopithecus rosalia* was considerably larger, covering the state of Rio de Janeiro (Kinzey 1982). However, given that the lion tamarin range is spread north to south along the Atlantic Forest, there may have been a chance for clinal differentiation. Our data provide no support for a clinal distribution of lion tamarins. The 2 geographically closest lion tamarins —*Leontopithecus chrysopygus and L. caissara*— are not sister taxa in any of our phylogenetic trees, and the 2 most geographically distant lion tamarins —*L. caissara* and *L. chrysomelas*— do not show the most divergence in phylogenetic relatedness or genetic distance.

Lion tamarin differentiation and distribution may be the products of Quaternary climatic fluxes (dry to wet) that caused forest contractions and expansions, because much of the Atlantic Forest consisted of refugia during the Quaternary (Whitmore and Prance 1987). Kinzey (1982) proposed 3 centers of endemism or forest refugia based on extant primate diversity: Bahia, Rio Doce, and Paulista. The centers are similar to those proposed by Müller (1973) based on herpetological data. The center Müller proposed is the Serra do Mar, which is divided into 3 subcenters: Pernambuco, Bahia, and Paulista (Fig. 8). Müller's Bahia subcenter subsumes Kinzey's (1982) Bahia and Rio Doce centers and the Paulista subcenter subsumes

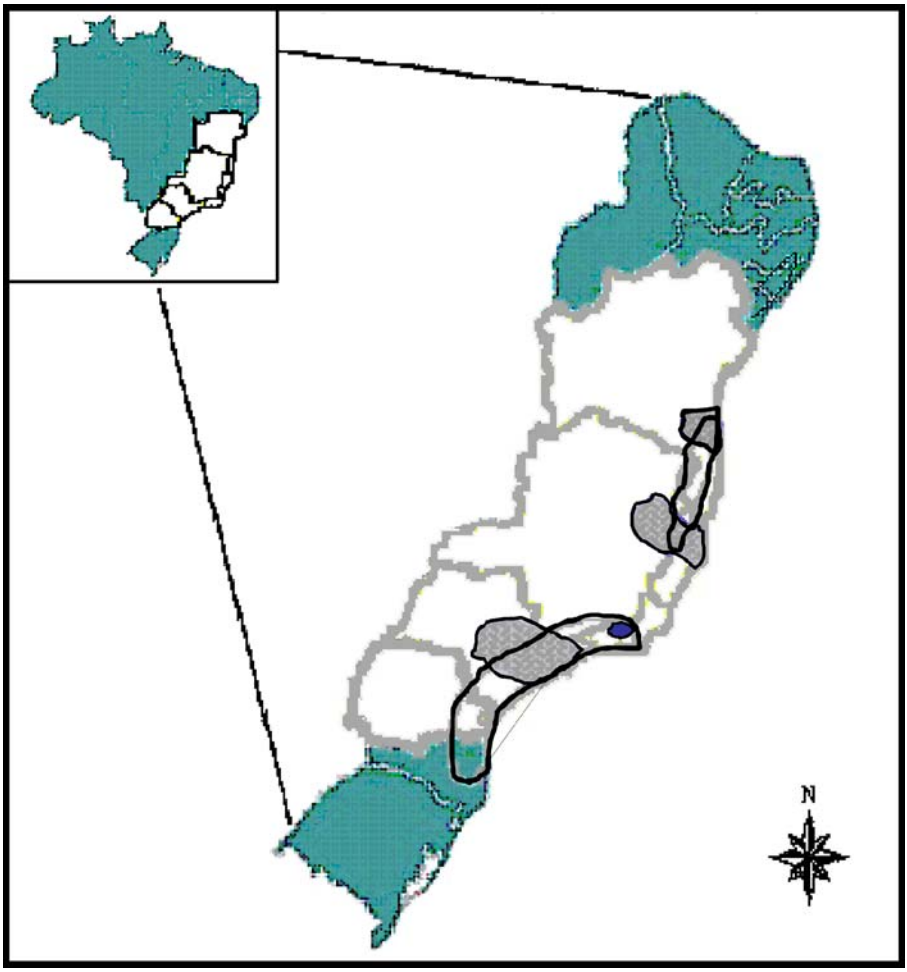

Fig. 8 Forest refuges along the Atlantic Forest Kinzey (1982); Müller (1973), and Jackson (1978) proposed (modified from Kinzey 1982). The outlined oval-like shape with no shading are refuges Müller (1973) proposed and, from north to south, the refuges are as follows: Bahia, Paulista. The outlined oval-like shape with a checker pattern are refuges Kinzey (1982) proposed and, from north to south, represent the Bahia, Rio Doce, and Paulista refuges. The oval with solid shading is a refuge Jackson (1978) proposed called the Orgãos refuge.

Kinzey's Paulista center. While many argue against referring to extant endemic locations as historical endemic isolates (Bush 1994; Haffer 1993; Whitmore and Prance 1987), Kinzey (1982) proposed that *Leontopithecus* was distributed throughout the Atlantic Forest and diversified allopatrically as a consequence of forest contractions as follows: *Leontopithecus chrysomelas* in Bahia, *L. rosalia* in Rio Doce, and *L. chrysopygus* in Paulista (Fig. 8).

The mitochondrial phylogenetic separation of *Leontopithecus chrysomelas* underscores that Bahia was a forest refuge that isolated *L. chrysomelas*. In addition, the position of *Leontopithecus chrysomelas* as the most ancestral of the lion tamarins suggests that Bahia was the first lion tamarin refuge to be isolated, and may even suggest that *Leontopithecus* arose in the northern part of its distribution and moved south.

Given the similar degree of genetic divergence among *Leontopithecus caissara*, *L. rosalia,* and *L. chrysopygus* and their geographic proximity, the lion tamarins may have existed in 1 refuge, perhaps corresponding to Müller's Paulista subcenter, which is a north/south extension of Kinzey's Paulista center and includes the region that these 3 taxa live in today. The phylogenetic data suggest that this 1 refuge subsequently divided, with the southern portion containing *Leontopithecus caissara,* though this division does not correspond to any hypothesized primate refuge. After the area with *Leontopithecus caissara* separated, the Paulista refuge may have divided further to separate *L. chrysopygus* from *L. rosalia*. As Natori (1989) suggested, the division may have resulted from the Orgãos refuge formation (Jackson 1978; Fig. 8), which better reflects the present location of *Leontopithecus rosalia* than does the Kinzey (1982) Rio Doce refuge.

Future studies on the diversification and taxonomic status of the lion tamarins would benefit from increased sampling and broader nuclear DNA phylogenetic analysis of all *Leontopithecus* taxa.

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